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A Practical Guide to Inoculation with Arbuscular Mycorrhizal Fungi in Ecological Restoration

SERDP Project RC-2330

JUNE 2017

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TABLE OF CONTENTS

LIST OF FIGURES, TABLES, AND BOXES IN SEQUENTIAL ORDER

SUPPLEMENTS

Table S1. Comparing the mycorrhizal responsiveness of many different species across succession

Box S1. Comparisons of the different ways to sterilize soil

SUMMARY

Despite billions of dollars being used for grassland restorations, current restoration practices fail to restore native grassland diversity, composition and function. We present evidence that the success of restorations can be improved by reintroduction of components of the native plant microbiome. In particular, we find that a group of root symbionts, arbuscular mycorrhizal (AM) fungi, play critical roles in grasslands community structure and that these fungi are sensitive to anthropogenic disturbance. Greenhouse assays demonstrate that late successional prairie plant species are more dependent on AM fungi and more sensitive to AM fungal identity than early successional plant species or non-native invasive plant species. Field inoculation assays show that reintroduction of the native AM fungi into disturbed landscapes can facilitate establishment of conservative plant species that are often missing from standard restorations, thereby improving plant diversity, accelerating succession and enhancing restoration quality. Early establishment of late successional grassland species can suppress non-native weedy plant species, thereby reducing management costs. We present and discuss the costs and benefits of approaches to isolate, culture, and reintroduce native AM fungal communities.

BACKGROUND ON RESTORATION, PLANT ECOLOGY AND SOIL MICROBES

Despite billions of dollars being used for restoration of native grassland plant communities in the United States alone (BenDor et al. 2015), restoration outcomes vary widely and it is not clear why (Figure 1). Generally, restorations have far lower plant species diversity compared to nearby remnant grasslands diversity (Kindscher and Tieszen 1998, Sluis 2002, Martin et al. 2005, Polley et al. 2005, Piper et al. 2007, Middleton et al. 2010), and plant species richness, especially forb species, can decline over time (Baer et al. 2002, Grman et al. 2013). In addition, the plant species seeded into a restoration upon its establishment are often not well represented in its resulting plant community (Grman et al. 2015). Although management strategies and site histories are indicators of restored plant community composition (Grman et al. 2013), much of the variation in restoration outcomes remains unexplained. The variation in restoration outcomes is likely due to restoration protocols that focus primarily on the plant community rather the establishment of other important grassland organisms. Here, we will argue that the focus on reintroduction of plants without re-establishment of native soil communities may be limiting restoration success.

Figure 1. Late successional remnant prairies (bottom) can have higher plant diversity, functional diversity, and average Coefficient of Conservatism than comparable prairie restorations that are seeded with a diverse, 54 species seed mixture (two upper photos). Whether dominated by grasses, forbs, or both, many restorations are largely comprised of early successional plants that have a low responsiveness to the presence of arbuscular mycorrhizal fungi, such as Monarda fistulosa, Elymus canadensis, Rudbekia hirta, *and* Solidago canadensis. *Plants that are more common in undisturbed prairies, are generally more responsive to arbuscular mycorrhizal fungi, such as* Echinacea pallida, Amorpha canescens, *and* Silphium terebinthinaceum. *Photos taken by Jim Bever and Liz Koziol.*

Accumulating evidence identifies the soil microbiome as an important driver of plant community composition. Experiments and field studies have identified that soil microbes play important roles in plant local adaptation (Schultz et al. 2001, Johnson et al. 2010, Bever 2015), coexistence (Bever et al. 2015), relative abundance (Klironomos 2002, Mangan et al. 2010), succession (De Deyn et al. 2003, Bauer et al. 2015), and invasions (Callaway et al. 2004, Pringle et al. 2009, Vogelsang and Bever 2009). Given this growing realization that soil microbiomes can structure plant communities, it is logical that successful restoration of native plant communities may require re-establishment of the native soil microbiome. Although many components of the soil microbiome might be important to plant and ecosystem function (Bever et al. 2013), plant symbionts are obvious first candidates to aid restoration.

Terrestrial plants have critical and long-standing relationships with root symbionts, including arbuscular mycorrhizal (AM) fungi which are present in fossilized roots of some of the first plants that colonized land nearly 300 million years ago (Remy et al. 1994). This symbiosis between plants and AM fungi represents one of the oldest and most widespread mutually beneficial interactions on earth, and most extant plant species continue to benefit from this mutualism. Plants rely on AM fungi to collect and deliver phosphorus and other nutrients that limit plant growth, which the fungi do in exchange for carbohydrates from the plant. AM fungi infect host plant roots and then send threadlike hypha beyond the root zone, mining nutrients from the soil and transporting them back to their host plant. These fungi can also provide plants with non-nutritional benefits by alleviating environmental stressors such as drought (Davies et al. 1993, Koziol et al. 2012), and diminishing host susceptibility to herbivory (Bennett and Bever 2007, Middleton et al. 2015). Beyond benefitting the growth of a plant host, AM fungi confer other ecosystem services. For instance, AM fungi can reduce soil erosion by increasing soil aggregate stability, both by physically enmeshing soil particles and also by binding particles together through production of a sticky glycoprotein called glomalin (Wright and Upadhyaya 1996, Rillig and Mummey 2006). Because of the diverse benefits that plant communities can either directly or indirectly receive through associating with AM fungi, the reintroduction of these soil microbes has the potential to promote native plant growth in grassland restorations while improving soil health and ecosystem quality.

During the last 15 years restoration scientists working in grasslands have experimentally tested the response of plants to inoculation with native soil microbes in general, and native AM fungi in particular (e.g. (Bever et al. 2003, Koziol and Bever 2016b). Inoculation with soil microbes by prairie restoration practitioners has spread to the private sector, where restoration seed companies are offering mycorrhiza, rhizobia, or other soil microbial amendments for purchase (Prairie Moon Nursery 2017). Some native plant restoration research has demonstrated dramatic results from the addition of AM fungi, including increased plant diversity and shifts in plant community composition from non-desirable to desirable plant species (Koziol and Bever 2016b) as well as potentially dictating the transitions between plant community types (Wubs et al. 2016). However, inoculation does not always result in the improvement of grassland restorations (e.g. commercial inocula treatment (Middleton et al. 2015)).

OBJECTIVES OF GUIDE

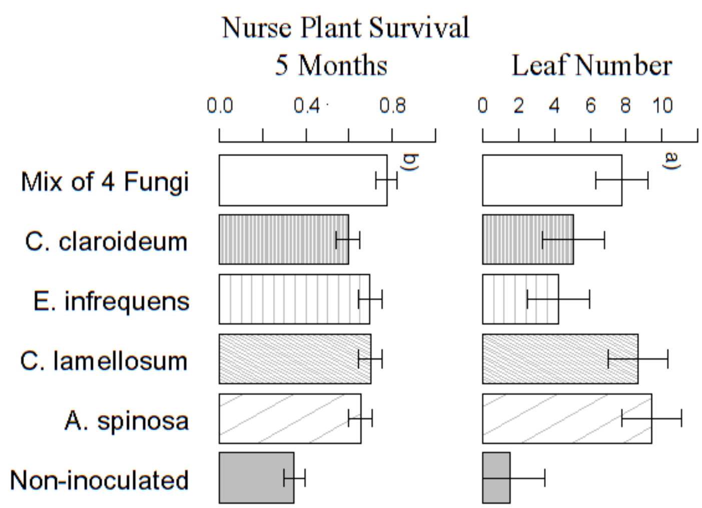
The objective of this guide is to summarize what is known about inoculation treatments with AM fungi to determine when inoculation with AM fungi is likely to enhance plant establishment, growth and survival in restorations. We will begin by reviewing the findings of restoration ecologists and discussing cases where reintroduction of AM fungi has improved

restoration outcomes. We will share our collective knowledge and experience on the use of AM fungi in restorations. Specifically, we will discuss our understanding of why inoculation can enhance restorations and when inoculation in restorations are likely to be beneficial. We outline different inoculation techniques, AM fungal inocula sources, and discuss their costs and benefits. Finally, we will also outline how restoration practitioners might choose one of the AM fungal inocula and how best to incorporate inocula into grassland restoration. While most of our work has been in the tallgrass prairie, we expect that the underlying community dynamics, and the value of inoculation with native AM fungi, will be similar for other native grassland communities.

WHY INOCULATE WITH AM FUNGI? THE POTENTIAL BENEFITS TO GRASSLAND RESTORATION

To illustrate the potential benefits of inoculation with AM fungi in native grasslands restoration, we describe the results from a recent experiment assessing the effects of five different native AM fungal inocula (Koziol and Bever 2016b). This study established prairie plants within an area with a history of agriculture that was maintained as mown lawn for several decades prior to planting. The turf in the area to be planted was weakened by spreading black plastic over the ground from March 1st –April 1st 2014. The experiment compared using individual and mixtures of species of AM fungi derived from prairies in Indiana as inocula. The AM fungi were introduced by planting inoculated seedlings (nurse plants) as fungal hosts; this technique is described in more detail below. Prairie plants inoculated with AM fungi were about 40% more likely to survive and grew three times larger than non-inoculated seedlings in the first year (Fig. 2a and 2b). These benefits spread to neighboring plants because plots inoculated with AM fungi had greater establishment from seed and higher coverage by desirable prairie plants, in addition to having lower weed coverage and more desirable, late successional prairie plant species (Figs. 3a-3c, (Koziol and Bever 2016b)). The benefits of initial inoculation can be amplified over time as successful establishment of long-lived late successional plant species can suppress invasive plants (Middleton et al. 2010) and thereby reduce future management costs.

Figure 2a. Nurse plant productivity (leaf number) was strongly improved with AM fungal inoculation although growth improvement was strongly dependent on AM fungal composition. Figure 2b. Inoculation improved nurse plant survival by nearly 40% during year one. Bars represent the average nurse plant survival (2b) and the number of leaves or tillers (2a) among plots inoculated with the six different fungal communities and error bars are standard error.



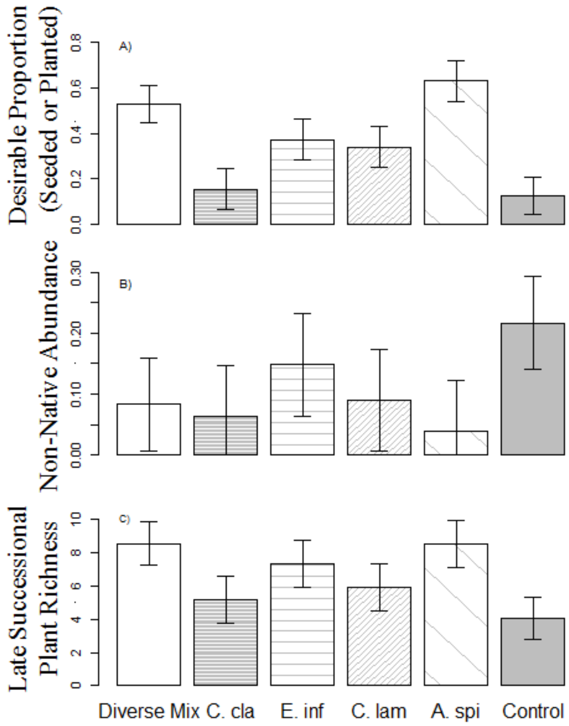


Figure 3A. Inoculation during year one resulted in increased desirable abundance (3A), lower non-native abundance (3B) and increased late successional richness (3C). We found inoculation with a diverse mix of native AM fungal species doubled desirable abundance and late successional richness and reduced by half the abundance of undesirable weeds relative to the control. Bars represent the portion of plot biomass that was desirable (3A) or non-native species (3B), and the total plot richness of late successional plant species (coefficient of conservatism of five or greater) (3C) among plots inoculated with the six different fungal communities and error bars are standard error.

Increased establishment of native species has also been observed in other independent inoculation experiments in North American grasslands (Bever et al. 2003, Middleton and Bever 2012, Middleton et al. 2015) and other grasslands in Europe, Asia, and elsewhere (Zhang et al. 2012, Wubs et al. 2016). While more work is needed to confirm benefits in all grassland types, we found consistent benefits across many independent grassland restoration attempts (Fig. 8) Inoculation with native AM fungi can promote resistance to herbivory (Middleton et al. 2015) and also improve soil aggregate stability and reduce erosion (Duchicela et al. 2012). Through improvement of native plant species establishment, re-introduction of beneficial mycorrhizal at restoration sites can produce higher quality restored habitat for wildlife and pollinators. We expect that the value of reintroduction of AM fungi to restorations will depend upon the context in which they are being used, including the land-use history of the site, the plant species planted, and the AM fungi chosen for inoculation.

When might AM fungal inoculation be most Beneficial?

Restorations occur in degraded sites that have been altered physically, chemically and biologically. The particular land use history of a restoration site may determine where mycorrhizal inoculations are likely to improve restoration success. Although we lack a detailed understanding of the natural history of most soil microorganisms, including AM fungi, it is well understood that soil microbial communities generally (Fierer et al. 2013), and AM fungi in particular, are generally negatively impacted by anthropogenic disturbance (Egerton-Warburton and Allen 2000, Oehl et al. 2003, Moora et al. 2014).

The net effect of major disturbances such as conventional agriculture, which combines multiple individual stressors, is that soils have significantly degraded AM fungal communities that may require microbial restoration. Mechanical disturbance such as tillage is highly destructive to

soil organisms, and microbial biomass may require decades to recover after its cessation (Bach et al. 2010). Tillage decreases AM fungal density and causes a shift in community composition where sensitive species are replaced by weedy fungi (Oehl et al. 2003). AM fungal density of the weedy fungi can recover quickly, but the sensitive species are effectively lost from the system. Applications of chemicals, such as glyphosate and/or fungicides, can reduce the viability of AM fungi and other beneficial soil microbes such as *Rhizobia* (Druille et al. 2013, Druille et al. 2015). Agricultural lands that receive repeated fertilization have been shown to be dominated by less beneficial, weedy AM fungal species, as plant investment in beneficial AM fungi declines (Johnson 1993, Ji and Bever 2016). Few studies have investigated the effects of genetically modified (GMO) plants on soil microbes, but one study found evidence that Bt modified corn can reduce mycorrhizal species richness in soils (Cheeke et al. 2012).

As many species of AM fungi only disperse through hyphal extension, and native grasslands are often not available nearby as sources of natural colonization, AM fungal communities are very slow to recover following abandonment of agriculture. We illustrate this problem with recent analyses of AM fungal composition of prairies and post-disturbance old fields of Illinois and Missouri (Fig. 4). We find that many AM fungal taxa are relatively abundant in

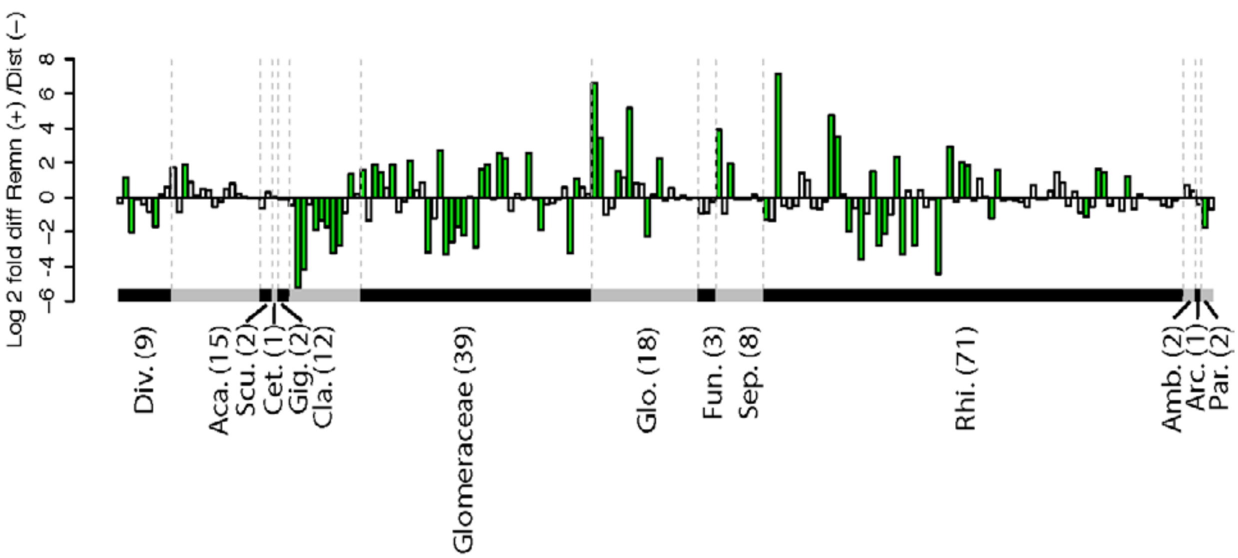


Fig. 4. Differential abundance of operational taxonomic units (OTUs) from AM fungal taxa in remnant prairies and nearby post-agricultural old fields across Illinois and Missouri. Taxa shown are genera except 39 OTUs that could only be attributed to the family Glomeraceae. The taxa are ordered by phylogenetic relationships (Redecker et al. 2013), with each bar representing the log difference in rRNA gene sequence abundance for each OTU either occurring in remnant or post-agricultural sites and significant differences are represented by filled bars. Thirty four OTUs were abundant in remnants and sensitive to disturbance (positive values) while twenty four OTUs had increased abundance with disturbance (negative values) (House and Bever 2017). Genus abbreviations: Div: Diversispora, Aca: Acaulospora, Scu: Scutellospora, Cet: Cetraspora, Gig: Gigaspora, Cla: Claroideoglomus, Glo: Glomus, Fun: Funneliformis, Sep: Septoglomus, Rhi: Rhizoglomus, Amb: Ambispora, Arc: Archaeospora, Par: Paraglomus.

undisturbed prairies and are sensitive to disturbance, while other weedy AM fungal taxa accumulate with anthropogenic disturbance (House and Bever 2017).

Another consequence of anthropogenic disturbance and a changing landscape is plant invasions. Several studies have demonstrated strong negative effects of plant invasions on AM fungal density and community composition (Hawkes et al. 2006, Pringle et al. 2009) which have negative effects on native plant establishment and growth (Pringle et al. 2009, Vogelsang and Bever 2009), and on soil aggregate stability (Duchicela et al. 2012). It is possible that these effects on AM fungal communities can persist long after an invasive species has been removed (Lankau et al. 2014). More work is needed to understand how often legacy effects occur and their duration, but it is likely that inoculation with AM fungi will improve restoration outcomes where invasive species with known negative effects on AM fungal communities occur.

Degradation of mycorrhizal fungal communities can substantially inhibit native plant establishment, with one study finding that as many as 50% of seeded species did not establish after soil mycorrhizae are disturbed and/or inhibited (Knappová et al. 2016). As expected from the slow recovery of AM fungal communities from anthropogenic disturbance, inoculation with AM fungi has been shown to improve establishment of native grassland species in abandoned agricultural fields, mown lawns, road cuts, and pastures (Bever et al. 2003, Middleton et al. 2015, Koziol and Bever 2016b). Dominance of non-native plant species on the landscape may be a good indicator of the value of inoculation (Vogelsang and Bever 2009, Duchicela et al. 2012). Reintroduction of AM fungi may be a less urgent need where a diversity of native grassland species has been established (Koziol et al. unpublished).

WHICH PLANT SPECIES BENEFIT MOST FROM AM FUNGAL INOCULATION?

Many plant species have been shown to benefit from mycorrhizal fungi, including hundreds of grasses, composites and legumes. Plant family or genus is often a good predictor of whether a given species will respond to AM fungi (Hoeksema et al. 2010, Reinhart et al. 2012). For instance, most *Carex* species are non-mycorrhizal (Miller et al. 1999) and therefore are not expected to benefit from re-establishment of native mycorrhizal fungi.

Recent work in prairies has indicated that plant successional stage may be the most reliable predictor of plant response to AM fungi. Fast growing early successional plants are less dependent on AM fungi, and slower growing late successional plants are highly dependent on AM fungi (Fig 5, (Koziol and Bever 2015), see Appendix S1 for a list of the mycorrhizal response of tallgrass prairie species). In addition, the growth of late successional plant species is more sensitive to changes in AM fungal species than early successional plants (Koziol and Bever 2016b). Therefore, when planting a restoration post-disturbance, late successional plant species are likely to be particularly sensitive to alterations in the AM fungal community that resulted from disturbance. Late successional plant species, including *Amorpha canescens*, *Eryngium yuccifolium*, and *Sporobolis heterolepis*, are also highly valued in restorations (Bauer et al. 2017) so inoculation with native AM fungi may be important for increasing grassland restoration quality. Consistent with these expectations, reintroduction of native AM fungal communities and whole soils has been shown to: 1) improve establishment of late successional, highly conservative plant species (Hoeksema et al. 2010, Reinhart et al. 2012, Middleton et al. 2015, Koziol and Bever 2016b), 2) substantially accelerate succession (Middleton and Bever 2012, Wubs et al. 2016), and 3) improve restoration quality in both US and European grasslands (Middleton and Bever 2012, Koziol and Bever 2016b, Wubs et al. 2016).

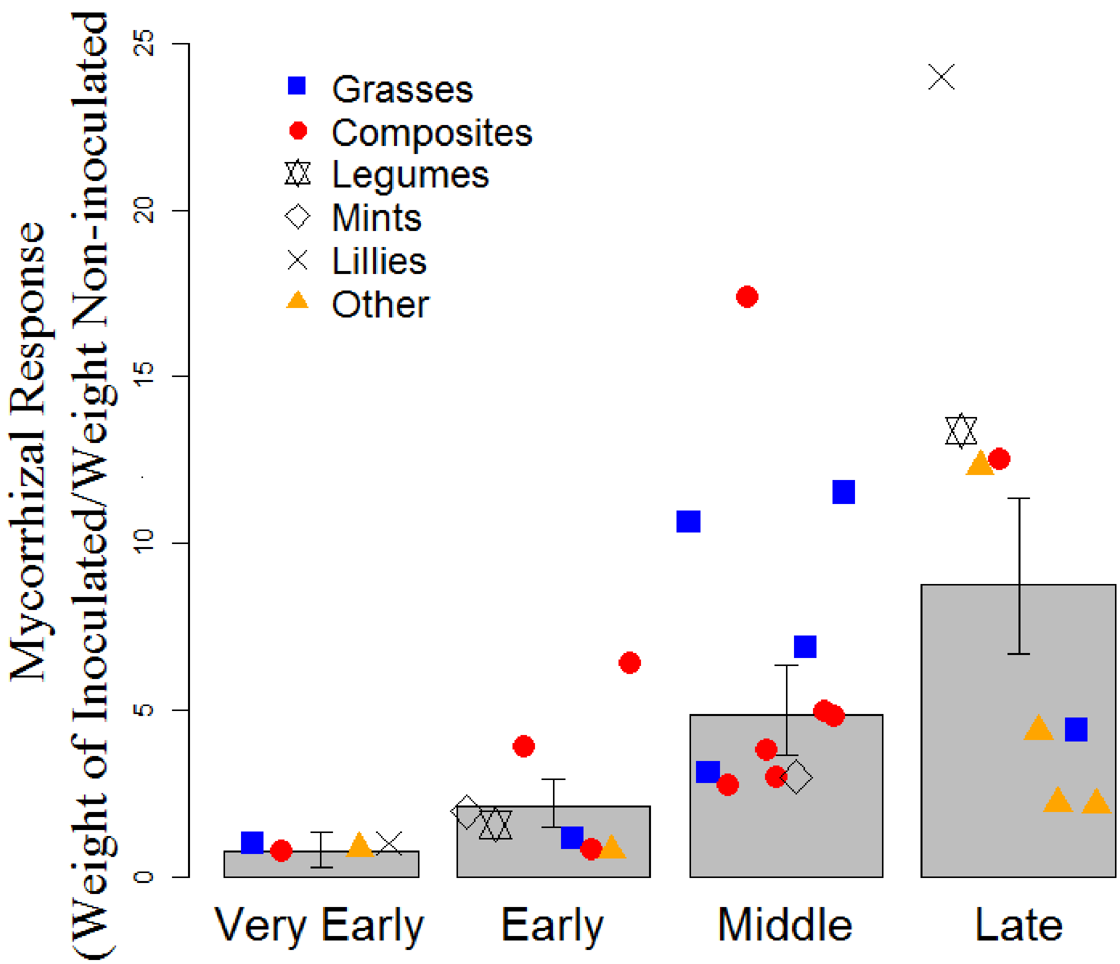
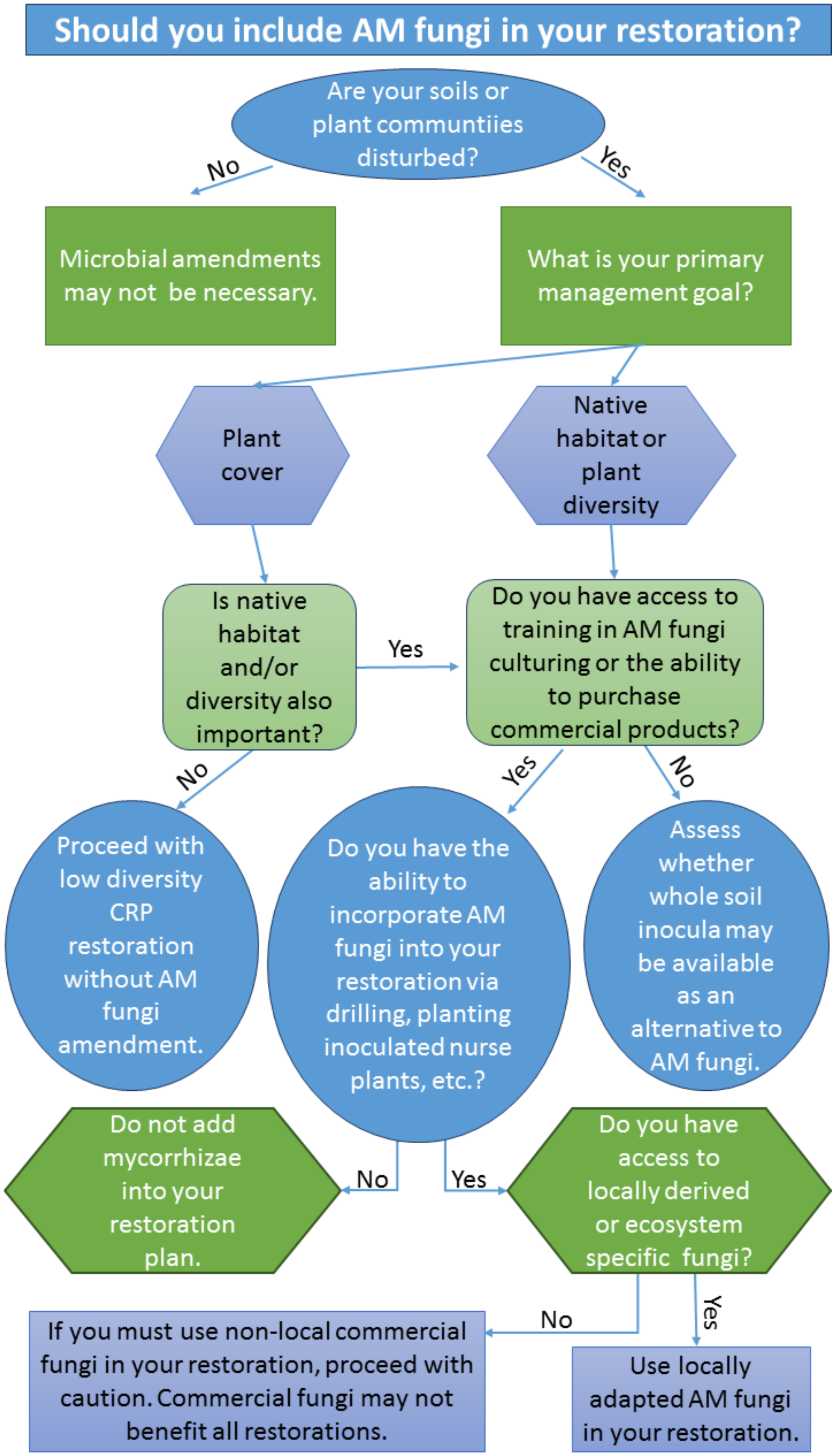


Figure 5. Mycorrhizal responsiveness is stronger for highly conservative plant species most common to undisturbed landscapes, while plants of low conservation prioity tended to be less responsive to mycorrhizal fungi. These results indicate that plants of low conservation priority may be most likely to establish in disturbed soils that have weakened mycorrhizal fungial communities. This effect was consistent across plant family (different colored and shaped symbols). Gray bars represent the mean mycorrhizal responsiveness (weight of inoculated/weight of non-inoculated plants) for four different successional stages. Error bars represent variation between plant species in each successional stage.

In summary, deciding whether or not to include AM fungal inocula in your restoration is dependent on how, when and why AM fungi may improve your restoration. The desired plant community and management goals for the restoration can drive this decision. To facilitate this decision, we summarize this section with a flow chart (Figure 6). In the following sections, we will discuss in detail the microbial collections and inoculation techniques that we have found useful for restoration.

Figure 6. A flow chart for assessing whether AM fungal inoculation should be used in a restoration project.



TYPES OF AM FUNGAL INOCULA

Once you have decided that you should inoculate your restoration with AM fungi, what inocula should you use? There are several types of inocula that can introduce native AM fungi, including whole soil inoculations, trap culture inoculation, and inoculations with particular species of AM fungi. Each of these inoculum types has its own strengths and weaknesses as we describe briefly in the following table (Table 1, below) and discussion.

Table 1. Comparisons of the different types of microbial inoculum

Types of Microbial Inoculum

Whole Soil Inocula from Reference Ecosystems	Trap Cultured Microbes	Mycorrhizal Cultures
PROS: • Contains the complete array of the soil community including AM fungi, beneficial bacteria pathogens, soil-dwelling insects, nematodes, and even seeds or other plant propagules. • Easiest way to find locally adapted, reference ecosystem microbes with the lowest associated cost.	**PROS:** • The trap culture method is a way to increase a small volume of whole soil inocula into a larger volume of whole soil inocula. This results in less disturbance of reference ecosystem soils. • Early iterations of trap cultures contain the complete array of the soil community including AM fungi, beneficial bacteria, pathogens, soil-dwelling insects, and other soil biota.	**PROS:** • Contain beneficial mycorrhizae without harmful plant pathogens. • Many types of commercial mycorrhizae can be easily purchased from a garden store or online. • Recent studies suggest that reference ecosystem mycorrhizae alone may be as beneficial as whole soil inocula, which may reduce the need to disturb remnant grassland soils.
CONS: • Collecting whole soils is destructive to remnant habitats which may be sparse. • Large volumes of whole soil inocula may be difficult or impossible to obtain. • Whole soil inocula may contain pathogens that are harmful to plants. • Effective inoculation rates reported in the literature range from 150-10,000 gallon per acre and lower rates have not been tested.	**CONS:** • Require sterile growing environments and greenhouse or equivalent to avoid contaminating the microbes. • Trap cultures of microbial communities may change over time and it is difficult to determine what is being cultured. For example, microbial species decline or the culturing of weedy species is possible using trap cultures.	**CONS:** • Require training and sterile growing environments to culture fungi or funds to purchase products. • Commercial mycorrhizae that are not locally adapted to a restored community type or location may be ineffective. • The suggested inoculation rates of commercial inocula are largely ineffective and successful inoculation rates have not yet been tested.

Whole soil

Whole soil is as simple as it sounds; it is inoculating with intact rhizosphere soil containing the complete array of the soil community including AM fungi, beneficial bacteria such as nitrogen fixing rhizobia, pathogens, soil-dwelling insects, and nematodes as well as plant roots. Using whole soil from a reference, undisturbed remnant grassland has been shown to be more beneficial to target plant growth and community richness than disturbed whole soils collected from old fields (Bever et al. 2003, Ji et al. 2010, Middleton and Bever 2012). This pattern has also been observed using reference ecosystem whole soils within heathland restorations (Wubs et al. 2016). Conversely, when microbial inocula from whole soil excluded AM fungi, plant growth did not increase (Ji et al. 2010). Together with other comparisons, this suggests that AM fungi may be the most important microbes driving plant benefits from whole soil inocula.

While incorporating whole soils into restorations may be one of the simplest methods to improve restoration success, it is also highly destructive of remnant sites because literally tons of whole soil could be required to establish native plant communities in restorations. Additionally, in much of the former grassland range, whole soil from the native ecosystem is unavailable or limited. In some states, less than 0.01% of tallgrass prairie remains; collecting any volume of soil from these highly threatened ecosystems is highly restricted and removing large volumes of soil are not feasible. Rarely, harvesting whole soil can be done in an ethical way, for example if a remnant tract of prairie is slated for destruction, negotiating the harvest of the topsoil for use in restorations would be a way to help mitigate the loss of the remnant plant community.

Trap cultures

Trap cultures are a way of 'bulking up', or increasing the biomass of the AM fungal community present in whole soil before it is used as inoculum in a restoration. Using this method, the AM fungi, as well as other plant dependent soil microbes, are amplified by growing with plant hosts in a pot and this allows their propagation for future use. To begin trap cultures, soil is collected from the rhizosphere of the reference ecosystem (i.e. from an undisturbed ecosystem of the type that you are trying to restore). This soil is mixed with sterilized growing media such as sterilized soil, calcined clay, or potting soil, at a 1 to 10 ratio of whole soil to sterilized background growing media. It is essential to use sterilized background growing media to ensure cultivation of soil organisms from the whole soil reference ecosystem and not organisms from the background medium (See Box S1 for information on soil sterilization techniques). Host plant seedlings grown in sterile growing media are then planted into pots filled with this whole soil mixture and grown under high light in a greenhouse for a growing season (5-10 months). During this time, AM fungi infect the plant roots (Fig. 7 top), proliferate and then produce asexual spores (Fig. 7 bottom) that represent their dormant structures. Overwintering (in continental climates) or oversummering (in Mediterranean climates) may be important to break dormancy of some AM fungal species. The AM fungal composition can be changed by the host plant identity (Bever et al. 1996, Eom et al. 2000) and pathogens can build up on single plant species (Bever 1994, Bauer et al. 2015), so we recommend using a diverse group of plant species that are native to the reference site in each pot when establishing trap cultures.

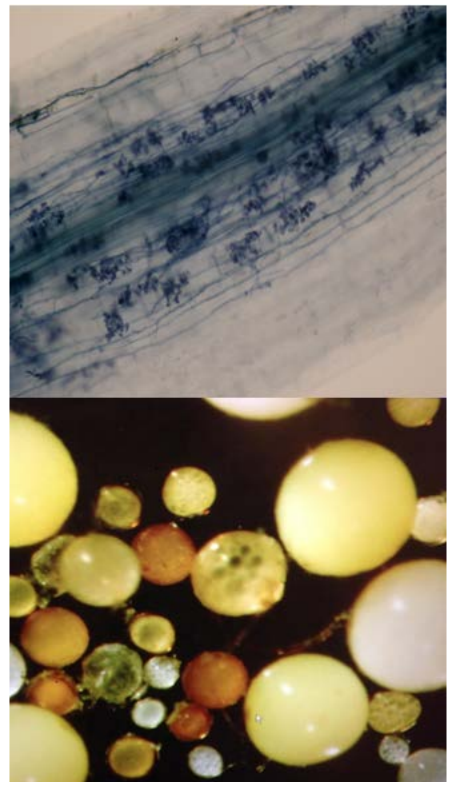


Figure 7. Above. Long, slender fungal hyphae and highly branched arbuscules of AM fungi are stained blue inside of a plant root. Below. AM fungal spores of 9 different AM fungal species. Photos by James Bever and Liz Koziol.

A benefit of trap cultures is that after harvest they can be used to inoculate additional rounds of cultures and these repeated rounds of whole soil trap culturing can be pursued indefinitely. Thus, this method is highly beneficial in that soil collection from the reference ecosystem only occurs once. However, this method also has limitations. The ability to sterilize soil and to grow appropriate host plants in a greenhouse environment that is free from contamination of other

microbes may be challenging for restoration practitioners (http://extension.psu.edu/pests/plant-diseases/all-fact-sheets/sources-of-disease-in-greenhouses). While the diversity of the microbial community may be viewed as a benefit, it may also complicate the attribution of benefit to particular microbial or AM fungal species. Moreover the composition of the microbial community may not be consistent between pots or across time. Some mycorrhizal fungi may require many months to years for sporulation to occur; one study found that mycorrhizae may not be abundant until after three trap culture cycles (Stutz and Morton 1996). Repeated culturing may also result in loses of AM fungal diversity, with some cultures becoming dominated by a single, weedy species that may not be particularly beneficial (Trejo-Aguilar et al. 2013). Therefore trap cultures may not be a reliable way to introduce a diverse array of AM fungal species into restored soils. Additionally, the added facilities cost and time required to produce trap-cultured soil may not be feasible for restoration practitioners without a partnership with or training by a microbial researcher.

Pure arbuscular mycorrhizal fungi cultures

Mycorrhizal cultures contain only living propagules of single or multiple AM fungal species. These cultures do not include the full suite of soil microbes, and should not contain soil pathogens, nematodes or insects, found in whole soil or trap culture inoculations. Methods to propagate AM fungi can be found on several prominent websites (INVAM, mycorrhizas.org). Here, we provide a brief description (Box 1). As use of laboratory equipment (i.e. microscopes, centrifuges, sterile greenhouse spaces) as well as training by a mycorrhizal biologist is required, cultivation of mycorrhizal fungi is likely to be beyond the scope of most restoration practitioners.

Box 1: How to culture AM fungi

1. Collect whole soil from relevant reference system.
2. Extract spores from the soil
3. Microscopically collect spores and assort them into groups by species/morphotype using a 75 X dissecting scope
4. Inoculate the roots of a high quality plant host with a single species of AM fungi as it is being transplanted into sterilized soil
5. Allow the plant and fungi to grow for 3-9 months.
6. Check viability of around 50 cm^3 of each culture by spore extraction. Suspend the spores in 60% sucrose, centrifuge for 1 minute, then pass the sample over a 38 micron sieve.
7. Mix single species cultures into a diverse species mixture prior to being used as inoculation.

How to evaluate commercial arbuscular mycorrhizal fungi cultures

AM fungi are not equal in their effects on host plant growth and because of this the source of AM fungal inoculum appears to be a critical determinant of the effectiveness of mycorrhizal cultures in restorations. Many AM fungal species that are amenable to culturing are generalists, or “weedier” species that may be less beneficial to host plants. For example, the fungi that proliferate with disturbance (Fig. 3) are more likely to grow well in greenhouse conditions with relatively high soil nutrient conditions and annual plant hosts than are the fungi that are sensitive to

anthropogenic disturbance. These weedy, easy to culture AM fungi, such as *Rhizophagus intraradices*, are well represented in commercial cultures of mycorrhizal inocula that can be commonly found at garden and hardware stores and some grassland seed growers. However, there is evidence that at least some of these products do not benefit, and may actually inhibit, the establishment and growth of desirable native grassland plant species in restoration settings (Paluch et al. 2012, Middleton et al. 2015). Commercial inocula also tend to be comprised of isolates that are not suited to local soil conditions and plant species, which could contribute to their poor performance. Meta-analyses of inoculation studies show that locally adapted AM fungal inocula are likely to benefit plant growth more than inocula that are dominated by easily cultured but weedy strains of AM fungi (Maltz and Treseder 2015, Rua et al. 2016). A mixture of AM fungal species is likely to promote the growth of a wider range of host plant species (van der Heijden et al. 1998, Vogelsang et al. 2006) and this can be particularly important for late successional grassland species (Figs 2 & 3) (Koziol and Bever 2016a, Koziol and Bever 2016b). We can only recommend using locally adapted reference ecosystem fungi in restorations at this time for two main reasons: 1) there is accumulated evidence indicating that generalist fungi are not beneficial in restorations and 2) the consequences of using commercial inocula are not well understood (Hart et al. 2017).

Choosing AM fungal Inocula

The best inoculum source to use in a restoration will depend on inoculum availability. For instance, whole soil collections are not likely to be possible in eastern tallgrass prairies and locally adapted mycorrhizal cultures are currently only available for ecoregions 222 and 251 (US Forest Service Ecoregions)(http://www.mycobloom.com). Few research studies have simultaneously compared different types of mycorrhizal inocula. In those that have, local whole soil inoculations or local soil trap cultures improve both desirable plant biomass (Emam 2016) and soil aggregate stability (Vogelsang and Bever, unpublished) compared to commercial AM fungal inoculum. However, even fewer studies have compared the effects of inoculation using locally sourced whole soil with inoculation using locally sourced mycorrhizal cultures.

In a multi-state comparison of the benefits of local native inocula, we found that whole soil and diverse AM fungal mixes produced similar benefits to late successional prairie plants. We established restoration experiments in Illinois, Kansas, and Oklahoma by inoculating eight mid- and late-successional prairie plant species with either whole soil from a nearby remnant prairie or cultured AM fungi from that same remnant. Across all sites, we found the average survival and plant growth was improved with both AM fungi and whole soil inoculations (Fig. 8). Inoculation with either AM fungi or whole soil promoted similar plant responses, although some plant species preferred whole soil and some preferred AM fungal inocula (Fig. 8). For instance, legumes often performed the best when inoculated with whole soil inoculations, likely because of the presence of other beneficial microbes including *Rhizobia* that were present in the whole soil inocula. In contrast, late successional forbs were often larger when inoculated with AM fungal cultures instead of whole soils, perhaps due to negative effects of soil pathogens in whole soil. We have expanded this data set to include nearly 20 plant species that are often desirable in restoration and have found that averaging across many plant species, inoculation with locally adapted AM fungi can improve

plant growth, survival, and establishment similar to locally adapted whole soils collected from a remnant reference ecosystem.

WHEN TO INOCULATE WITH AM FUNGI

Using either whole soil or AM fungal cultures in restorations requires careful consideration about when and how inoculation should take place in order to ensure its best chance for success. AM fungi are obligate symbionts that need to infect roots of effective hosts to ensure their growth and long term survival. In the absence of association with a growing host, AM fungal hypha and spores are vulnerable to desiccation and parasitism by bacteria and fungi, as well as consumption by worms and insects. Thus, mycorrhizal inocula are best introduced into a restoration with host plants or during spring seed application when plant roots will be quickly available for colonization. As many early successional and non-native weedy plants are poor hosts for mycorrhizal fungi (Vogelsang and Bever 2009, Lankau et al. 2014), the initial stages of a restoration represent a particularly sensitive period for inoculations. However, the early establishment of high quality mycorrhizas between growth-promoting fungi and desirable host plant species can generate a positive feedback that can accelerate restoration (Bever et al. 2012). Therefore, inoculation early in a restoration has the potential to have the greatest positive impact.

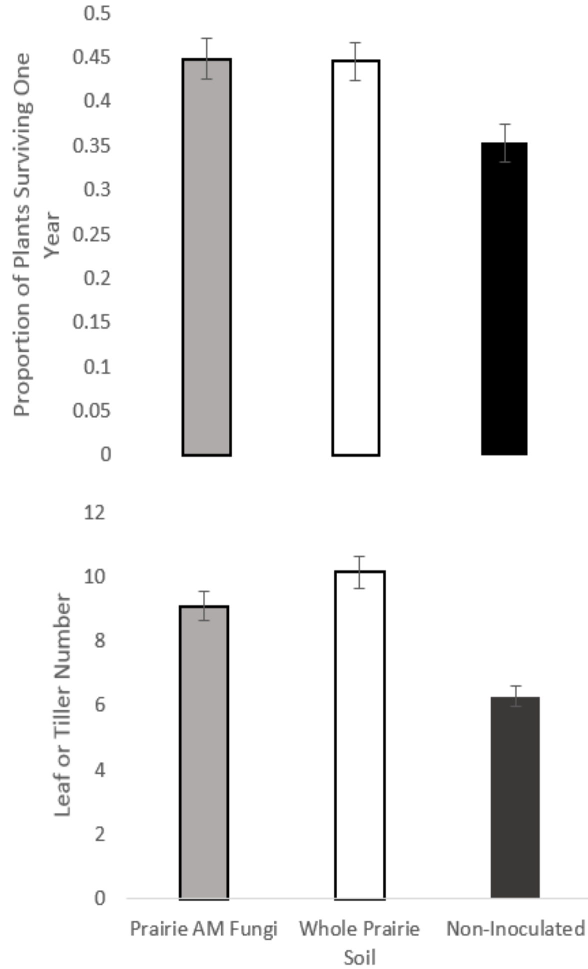


Figure 8. Survival (top) and growth (bottom) of prairie plants are significantly and simiarily increased by inoculation with prairie AM fungi or prairie whole soils suggesting that the prairie AM fungi are the most important component of the prairie soil community for native plant establishment (Koziol et al. unpublished).

HOW TO INOCULATE WITH AM FUNGI

Whole soil, trap cultures and mycorrhizal culture inoculation methods are very similar (Box 2, How to inoculate with soil microbes). Once an inoculum is selected, it can be distributed via broadcasting, hydroseeding, drilling, tilling, or inoculated nurse plants (Box 2)--each with associated costs and benefits.

Box 2. How to inoculate with soil microbes

Broadcasting Inocula

Broadcasting inocula over the surface of the soil is a simple and easy approach to dispersing AM fungi. Broadcasting means spreading the inocula over the surface of soil at the restoration site. This can be done by hand, with a garden seeder or by using fertilizer application equipment. We have participated in several unpublished studies using broadcast inocula, which failed. The main weakness of broadcasting is that microbes are not deposited belowground. AM fungal propagules on the surface of the soil are vulnerable to desiccation and solarization and are not likely to be in close proximity of plant roots. Therefore, broadcast methods that do not incorporate an additional layer that covers the inoculum or a method to introduce the inoculum belowground, such as tilling, are unlikely to succeed.

Tilling

Tilling inoculum into the first few inches of the ground is one way to help disperse the inoculum belowground. While this method is much more labor intensive than broadcasting inoculum, several studies have found that broadcasting mycorrhizal fungi or whole soil inoculum followed by tilling can be effective at improving desirable plant growth and richness (Bever et al. 2003, Emam 2016). These studies also used inoculation rates of 150-10,000 gallons of inoculum per acre, which is beyond the scope of many restoration projects in terms of cost, effort, and inoculum availability. Additionally, tillage further disturbs soil microbes including existing mycorrhizal fungi, beneficial bacterial, and soil nematodes. It is likely that this method is most effective if used immediately after major disturbance ends, for example, during the spring after a site is removed from tilled agriculture.

Hydroseeding

Hydroseeding is an approach to dispersing seed within a slurry of water and mulch, often via powerful pumps. AM fungal inocula can be included within the slurry to simultaneously distribute seed with its symbionts. Inoculation with this method has been effective in some field trials (Vogelsang and Bever, unpublished), but failed in others (Middleton et al., unpublished). As root and soil particles in mycorrhizal inocula can create mechanical problems for hydroseeding equipment, only a subset of potential inocula are compatible with this method. For example, many inocula are a coarse granular substance filled with many plant roots that may not be amenable to hydroseeding equipment. Moreover, even with the mulch, the inocula will likely have higher mortality and lower availability to active roots than inocula tilled into soil.

Seed dressing, seed inocula pellets, and seed/inocula drilling

Much of our knowledge on the effectiveness of creating mycorrhizal seed pellets as well as drilling inocula comes from agricultural systems as few studies have reported on their use in restoration. Many mycorrhizal inocula are comprised of fine sand, soil, calcined clay, or other inert growing media and would successfully be passed via a seed drill. Several studies have found that drilling inocula can result in effective mycorrhizal colonization (Cozzolino et al. 2013).

One study compared the effectiveness of broadcasting, tilling (raking), applying seed/inoculum pellets, and applying in a slurry and found that both tilling and the slurry resulted in the greatest mycorrhizal colonization whereas broadcasting and pellets did not result in increased mycorrhizal infection (Hayman et al. 1981). Many prairie seeds are large and/or have tufts that allow them to travel on the wind perhaps making them poor candidates for use in a pellet

formulation. Many seed pellet creation protocols involve the use of moist clay. While these methods have not been tested on AM fungi, using moist clay to adhere the seed and fungi may be problematic, as fungal spores germinate upon wetting. As the pellets are then dried before use, the mycorrhizae may die thereby rendering the pellets inert. We have found no studies reporting the use of inoculated seed pellets being used in grassland restorations and this method of mycorrhizal inoculation should be assessed in restoration.

The nurse plant method for microbial introduction

An approach that we have developed to maximize the probability of survival of AM fungal inoculum in a restoration is to introduce the fungi after formation of its mycorrhizal association with its host plant. Infecting potted seedlings with AM fungi before planting into a site allows them to act as nurse plants for the AM fungal inoculum and enhances the likelihood that the inoculum will remain viable in the field. However, planting inoculated host plants has several obvious costs. Growing host plants requires: 1) space and materials for soil sterilization and inoculation, 2) space to rear seedlings until time of planting, and 3) the labor of starting and planting nurse plant plugs. Additionally, the success of host plant establishment in the field is highly related to the weather during the first growing season after planting. However, we have found that inoculation increased the survivorship of conservative plant species even in years with suboptimal growing conditions (Middleton and Bever 2012, Middleton et al. 2015, Koziol and Bever 2016b).

The AM fungi inoculated on the nurse plant roots can spread to nearby un-inoculated plants, thereby spreading the benefits of inoculation through the restoration (Middleton and Bever 2012, Middleton et al. 2015, Koziol and Bever 2016b). Optimal planting designs of restorations would therefore depend upon the rate of spread of beneficial AM fungi. Benefits of inoculation to un-inoculated plants have been shown to spread over distances of up to two meters within a single growing season (Middleton and Bever 2012, Middleton et al. 2015). It is likely that the rate of spread of native AM fungi would depend upon the plant community context, as spread of native AM fungi would likely be inhibited by dominance of weedy plant species compared to restorations where there was high germination of late successional prairie plant species.

Suggested inocula application rates vary widely

As discussed above, the reported inoculation rates of soil microbes in experimental restorations have varied widely, with effective inoculation rates of 150 to 10,000 gallons of inoculum being used per acre. Commercial inocula suggest much lower inoculation rates of 5-60 lbs of mycorrhizal inocula per acre. However, many commercial inocula have also been shown to be ineffective (Maltz and Treseder 2015). Using planted nurse plugs, we have found inoculation rates of 32 gallons per acre (about 2/3 cup per square meter) to be effective (Middleton and Bever 2012, Koziol and Bever 2016b). Few studies have attempted to assess the effects of inoculation at lower inoculation volumes. These studies highlight a gap in our knowledge of effective inoculation rates. Native plant restoration would benefit from work aimed at assessing effective inoculation rates across different land use histories using different inocula sources.

HOW TO TELL IF INOCULATION WORKED

There are several ways to tell if inoculation was successful. The simplest method is to survey the plant community to observe the presence of plant species that are sensitive to soil microbes, primarily late successional plant species. We list species that consistently grow larger

with mycorrhizal fungi in the Supplementary Table S1. Similarly, we have also found many of these species are either unlikely to establish or have lower than expected establishment from seed in restoration projects that do not incorporate microbial inocula (Kindscher and Tieszen 1998, Piper et al. 2007, Grman et al. 2015, Koziol and Bever 2016b).

A second method to assess inoculation success would be to measure mycorrhizal infection and mycorrhizal composition in plant roots. However, these methods require training by mycorrhizal ecologists, laboratory access, and purchasing supplies to measure infection. A few companies will assess mycorrhizal colonization, however mycorrhizal consultation services are currently expensive, and gross measures of mycorrhizal colonization are not sensitive to changes in AM fungal composition. Soil health tests, such as PLFA, will not help to assess successful establishment of inoculum. Effectiveness of AM fungal composition has been confirmed from measures of AM fungal sporulation (Middleton *et al.*, 2015), environmental sequencing of specific AM fungal species could also be used (Pellegrino et al. 2012, Hart et al. 2015, Schlaeppi et al. 2016).

FUTURE DIRECTIONS FOR IMPROVEMENT OF GRASSLAND RESTORATIONS

We have outlined how reintroduction of one particular important component of the plant microbiome, AM fungi, can improve restoration outcomes. We suggest that this practice should be added to other standard grassland restoration management practices such as restoration of historical fire frequency and native herbivores. We expect these practices to be complementary, though to date, the use of native AM fungal inoculation has not been studied in conjunction with fire frequency and/or native herbivores. While the potential benefits of reintroduction of native AM fungi are strong (summarized in Table 2), major barriers remain in in the widespread implementation of this approach. We outline several logistical and conceptual challenges below.

Table 2. Pros and cons of inoculation with AM fungi in restoration

Pros	Cons
• Improved establishment of high quality plant species • Improved native plant establishment • Accelerated succession • Greater plant diversity • Greater resistance to invasion by non-native invasive plant species • Reduced vulnerability to soil erosion	• Availability of native AM fungal inocula is limited • AM fungal inocula can be expensive for large projects • Nurse plant method of inoculation is proven, but labor intensive • Expertise in development and evaluation of inocula is limited

The major logistical problem of using native AM fungi to inoculate grassland restorations involves the limited availability of appropriate inocula. While we have found that locally adapted mixtures of AM fungal strains are likely to be optimal for restoration of most conservative plant species (Middleton et al. 2015, Koziol and Bever 2016), to our knowledge, this type of inocula is only commercially available for the tallgrass prairie eco-region. Inoculating restoration at large spatial scales presents additional logistical challenges. While we have demonstrated that locally

adapted, native AM fungi can be effectively introduced into grassland restorations via the nurse plant method, more work is required to demonstrate the extent to which native AM fungal inocula can successfully be applied with mechanical approaches commonly used on large scale restorations such as hydro-seeding and drilling. Current evidence suggests that the most effective AM fungi for late successional prairie plant species are sensitive to mechanical disturbance, suggesting that they may be less tolerant to mechanical manipulation during inoculation than fungi that increase with disturbance (Fig. 4).

More generally, while the re-introduction of native AM fungi can improve grassland restoration, we do not know the extent to which successful restoration of native AM fungi will be sufficient to restore the original diversity and ecosystem services of undisturbed grasslands. The evidence that inoculation with native AM fungi accelerates restoration (e.g. Koziol and Bever 2016) is promising, but may not be sufficient for long-term stabilization of grasslands. Other components of the plant microbiome, such as plant pathogens, may also play major roles in species turnover during succession and maintenance of the original high diversity of grasslands (Klironomos 2002, De Deyn et al. 2003, Kardol et al. 2007, Mangan et al. 2010, Bauer et al. 2015, Bever et al. 2015). Whether restoration of native pathogens would improve stability of grassland restorations remains unknown.

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Supplemental Tables

Table S1. Table of plant species dependence on native AM fungi. Here we highlight the percent Indiana prairie plants and restoration colonizers were improved (percent greater than 0 (or inhibited by (percent less than 0)) Indiana prairie mycorrhizae relative to non-inoculated plants. We color code each plant species based on its conservation coefficient (CC) value (Swink and Wilhelm 1994). BLUE indicates species with of CC 6-10. YELLOW indicates species with moderate coefficient of conservation with CC 4-5. RED indicates non-native species or weedy species typically not included in seed mixtures. BLACK are species with low conservation concern (but still included in seed mixes) of CC 1-3. Generally, we found that later successional species that are difficult to establish in restoration are strongly responsive to prairie mycorrhizae, whereas non-native species and species of low conservation value tend to be less responsive to mycorrhizae.

Plant	Mycorrhizal Responsievness	Plant	Mycorrhizal Responsievness
Amorpha canescens	1200%	Liatris spicata	200%
Asclepias verticillata	1150%	Asclepias incarnata	180%
Parthenium integrifolium	1100%	Chamaecrista fasciculata	160%
Allium cernuum	1100%	Andropogon gerardi	150%
Verticillata altissimum	1080%	Bidens bipinnata	130%
Helianthus grosseserratus	1060%	Zizia aurea	60%
Eryngium yuccifolium	1010%	Rudbeckia hirta	50%
Coreopsis tripteris	1010%	Conyza canadensis	50%
Sorghastrum nutans	800%	Baptisia bracteata	40%
Schizachyrium scoparium	700%	Pycnanthemum virginianum	30%
Asclepias viridis	680%	Coreopsis palmata	30%
Echinacea pallida	660%	Panicum virgatum	30%
Veronicastrum virginicum	530%	Abutilon theophrasti	20%
Sporobolus heterolepis	500%	Solidago rigida	19%
Symphyotrichum laeve	460%	Monarda fistulosa	10%
Symphyotrichum novae-angliae	390%	Tradescantia ohiensis	-20%
Lespedeza capitata	390%	Penstemon digitalis	-20%
Silphium terebinthinaceum	370%	Carex vulpinoidea	-30%
Echinacea purpurea	340%	Carex scoparia	-30%
Lobelia cardinalis	340%	Rumex crispus	-30%
Asclepias tuberosa	340%	Koeleria macrantha	-30%
Helianthus occidentalis	330%	Carex tribuloides	-40%
Desmodium illinoense	330%	Chenopodium album	-40%
Bouteloua curtipendula	260%	Rumex patientia	-40%
Ratibida pinnata	215%	Panicum capillare	-40%
Heliopsis helianthoides	210%	Elymus canadensis	-50%
Silphium integrifolium	209%	Setaria viridis	-60%

Box S1 A HOW TO ON SOIL STERILIZATION

Soil that is used to grow AM fungal cultures, trap cultures, or to grow non-inoculated nurse and test plants should be sterilized of soil organisms including AM fungi prior to inoculation. Soil can be sterilized of AM fungi in a variety of ways that differ in cost ($-$$$$$$, where each $ represents a tenfold increase), quality, ease of use, and effectiveness. Generally, soil temperatures of 140 D F for one hour or longer are required to kill most soil pathogens. Autoclaving or steam aeration are the most effective but also the most expensive methods for sterilizing background soil.

Sterilization Method	**Technique**	**Benefits**	**Challenges**
Autoclave	Autoclave soil for 2 hours twice with a one day rest period in between	Kills most soil pathogens, pests, and AM fungi	Requires steam source $$$$$ and plastic container that is polypropylene (plastic code #5) to withstand autoclaving (up to 250 degree F) $$
Steam Aerator	Steam soil for 4 hours twice with a one day rest period in between	Kills most soil pathogens, pests, and AM fungi	Expensive equipment $$$$$
Electric Sterilization	Set machine to at least 170 degrees F for two hours twice with a one day rest period in between	Kills AM fungi Some seeds and other soil ogranisms can survive	Heated coils do not heat soil evenly. Wetting media slightly may distribute heat. $$$$
Conventional Oven	Bake soil at temperatures greater than 170 degrees F for 2 hours	Kills AM fungi	Must have access to gas or electric hook ups $$$
Outdoor Fire Oven	Place soil into containers positioned on top bricks above a well-built fire and monitor soil temperature	Kills AM fungi	Temperature must be monitored closely so as to reach 170 degrees F$

Fungicide	Apply 150 kg benomyl ha-1 equivalent to soil and allow dilute so that it can distribute in pots	Has been shown to effectively kill AM fungi; may not kill all AM fungi and other soil organisms	Benomyl is a possible human carcinogen and has a half life of 6-12 months in soil $$
Solarization	Build a box with a glass cover and monitor soil temperature to reach 170 degrees F for 2 hours	Temperatures *can* reach high enough to kill AM fungi.	Maintaining even temperature in soil can be difficult $